Biomarkers of Asthma

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Abstract

Biomarkers may be diagnostic of asthma, they may predict or reflect response to therapy or they may identify patients at risk of asthma exacerbation. A biomarker is most often measured in biologic fluids that are sampled using relatively non-invasive sampling techniques such as blood, sputum, urine or exhaled breath. Biomarkers should be stable, readily quantifiable and their measurement should be reproducible and not confounded by other host factors, or the presence of comorbidities.

However, asthma comprises multiple molecular endotypes and single, sensitive, specific, biomarkers reflecting these endotypes may not exist. Combining biomarkers may improve their predictive capability in asthma.

The most well-established endotypes are those described as Type2 and non-Type2 asthma. Clinical trials established the fraction of exhaled nitric oxide (FeNO) and blood eosinophil counts as key biomarkers of response to corticosteroid or targeted anti-inflammatory therapy in Type2 asthma. However, these biomarkers may have limited value in the management of asthma in real-life settings or routine clinical practise. Biomarkers for Type2 asthma are not well described or validated and more research is needed.

Breathomics has provided evidence to propose a number of exhaled volatile organic compounds (VOCs) as surrogate biomarkers for airway inflammatory phenotypes, disease activity and adherence to therapy. Analysis of urinary eicosanoids has identified eicosanoids related to Type2 and non-Type2 inflammation. Future clinical trials will be important in determining how exhaled VOCs or urinary eicosanoid profiles can be used to direct precision treatments. Their future clinical use will also depend on developing simplified instrumentation for biomarker analysis at the point-of-care.

Key Words; Asthma, Biomarkers, Endotypes

Introduction

In 2016 it was estimated that more than 339 million people suffer from asthma globally, and that asthma is the most common non-communicable disease among children, although most deaths occur in older adults [1]. More recently, estimates indicate that asthma is the second most prevalent chronic respiratory disease worldwide, with a prevalence of 3·6% (3·2–4·0) and accounts for nearly 0.5 million deaths globally each year [2].

Asthma is defined as a heterogeneous disease, usually characterised by chronic airway inflammation and airway hyperresponsiveness [3]. Patients have a history of respiratory symptoms including wheeze, shortness of breath, chest tightness and/or cough, with variable airflow limitation that may become persistent over time. It is estimated that 3-10% of asthma patients have severe asthma, defined as asthma that remains uncontrolled despite adherence with maximal optimised GINA step 4 or step 5 therapy (medium or high dose inhaled corticosteroid with a second controller, maintenance oral corticosteroid) and treatment of contributory factors, or worsens when optimised therapy is reduced [3].

Severe asthma is associated with increased mortality, hospitalisation and reduced quality of life, placing a physical, mental, emotional, social and work-place burden on patients in addition to an economic burden on healthcare systems [3, 4], and its effective management remains an unmet need.

Definitions;

Phenotype: The observable characteristics of a disease, such as morphology, development, biochemical or physiological properties, or behaviour [3].

Patients with an identified phenotype of asthma may share a cluster of clinical, functional and/or inflammatory features, without any implied common underlying mechanism or response to therapy. Examples include allergic asthma (eosinophilic and non-eosinophilic), non-allergic (eosinophilic, neutrophilic, mixed, paucigranulocytic) asthma, aspirin-exacerbated respiratory disease, asthma with obesity, asthma with persistent airflow limitation, late-on-set asthma [5] (Figure 1).

Endotype: A subtype of disease, defined functionally and pathologically by a distinct molecular mechanism or by distinct treatment responses [6]. Endotypes are not yet recognised in the GINA strategy document for asthma management and prevention [3].

Among patients with severe asthma there are likely to be several specific endotypes associated with divergent underlying molecular causes, and with distinct treatment responses. These endotypes may or may not align with clinical or inflammatory phenotypes [5, 7] (Figure 1).

The most well-described endotypes are those described as Type2 and non-Type2 asthma [8,9], reflecting the presence or absence of a central role for the Type2 cytokines, IL-4, IL-5 and IL-13, in airway inflammation [9]. Type2 asthma is responsible for approximately 50% of asthma and is characterised by more severe asthma, airway and systemic eosinophilia, responsiveness to glucocorticosterids (but not in all cases, especially older patients with more severe disease [10]) and responsiveness to targeted inhibitors of Type2 inflammation [9]. Type2 asthma occurs both with allergy, in which both $CD4^{+}$ Th2 helper T-cells and innate lymphoid type2 (ILC2) cells are sources of Type2 cytokines, and without allergy, in which Type2 cytokines are derived predominantly from ILC2 cells [11,12], (Figure 2).

On the other hand, non-Type2 asthma was reported to be associated with less severe disease, absence of airway and systemic eosinophilia, lack of responsiveness to glucocorticoids, and lack of responsiveness to targeted inhibitors of Type2 inflammation [9]. Cytokines and other potential mediators of non-Type2 asthma have not been well described, but may include IL-8, IL-17, inflammasome activation and IL-1β, as mediators of neutrophilic inflammation [5, 11, 12], and the epithelial-derived chitinase-like enzyme YKL-40 which may be involved in airway remodelling [13]. High bronchial tissue neutrophilia (with eosinophilia) was also recently associated with increased IL-17 and IL-22-positive tissue cells, increased CD4-positive cells, elevated serum IgE, more severe disease, increased exacerbations and greater OCS use, indicative a novel Type2/Th17 endotype [14].

However, to date, targeted inhibitors of TNFα, the IL-17 receptor subunit IL-17RA, and antagonists of the high affinity IL-8 receptor CXCR2 have not been clinically effective, bringing into question the role of neutrophils as a target for therapy in non-Type2 asthma [5]. Thus, mediator-targeted therapies are not currently available for patients with the clinical phenotypes frequently associated with non-Type2 asthma, which includes late on-set asthma, obesity-related asthma, smoking associated neutrophilic asthma and paucigranulocytic asthma [15]. However, 48-weeks of add-on therapy with the macrolide antibiotic azithromycin reduced exacerbation frequency and improved quality of life in patients with both eosinophilic and non-eosinophilic asthma [16] (Figure 2).

Bronchial thermoplasty, with ablation of bronchial smooth muscle and sensory nerves through application of localized thermal energy, is an option for patients with severe steroid refractory asthma with severe airway hyper-responsiveness ($PC_{20} < 0.25$) and frequent exacerbations despite absent or controlled airway inflammation [17] (Figure 2).

Biomarkers

A biomarker is a defined characteristic measured as an indicator of normal biologic processes, pathogenic processes or response to an intervention [18].

Biomarkers in asthma may reflect underlying airway inflammation and clinical presentation and be diagnostic of the disease, or they may predict or reflect response to therapy or they may identify patients at risk of asthma exacerbation. An ideal biomarker for asthma should be stable, easily measured, reflect underlying pathophysiology or treatment target, have a high sensitivity and specificity, be reproducible, inexpensive, and have prognostic, predictive and pharmacodynamic features [19].

Biomarkers of Type2 asthma

Eosinophils in sputum and blood

It is long-established that eosinophilic inflammation of the airways correlates with the severity of asthma, with activated eosinophils contributing to changes in bronchial epithelial integrity and function [20]. The current gold standard used to adjust inhaled corticosteroid (ICS) dose in asthma is sputum eosinophilia, typically measured as the percentage of eosinophils in induced sputum [21, 22]. Normalisation of the induced sputum eosinophil count reduced asthma exacerbations and admissions without the need for additional anti-inflammatory treatment [21, 22]. However, acute exacerbations occur in patients with all severities of asthma, and are usually treated with a short course of oral corticosteroids (OCS) to reduce inflammation and symptoms, or may be treated with maintenance OCS to control daily symptoms [23]. Patients with increased biomarkers of Type2 inflammation, sputum eosinophils, blood eosinophils and fraction of exhaled nitric oxide (FeNO), had improved response to OCS [23].

Although monitoring sputum eosinophils in asthma predicts exacerbations and improves management of asthma, the procedure for collecting sputum is somewhat invasive and not uniformly successful or widely available except in specialist centres [24]. Therefore less invasive, more widely available biomarkers are needed [25]. Potential examples of asthma biomarkers include FeNO, blood eosinophils, periostin and serum IgE, although these may not meet quality criteria for biomarkers [24].

Blood eosinophil counts may [26, 27, 28] or may not [29, 30] be a surrogate for sputum eosinophils in asthma. Blood eosinophil counts and FeNO, but not periostin, significantly correlated with sputum eosinophil percentages across the range of disease severity, mild to severe, and blood eosinophils were the best predictor and highly diagnostic for sputum eosinophils ≥2% or ≥3% [26, 27, 28]. However, others reported no correlation between blood and sputum eosinophils [29], suggesting they are biomarkers of different Th2 cytokine driven inflammatory processes in asthma, systemic (IL-5 dependent) and airway (IL-13 dependent) inflammation, respectively [30, 31], as described below. In this respect, the additive value of high FeNO (≥50 ppb) and blood eosinophils (≥500 cells/µl) for predicting wheeze and reduced lung function was confirmed [31].

Further, despite many cross-sectional, and some longitudinal studies, indicating that blood eosinophil counts are predictive biomarkers of asthma exacerbation rate [32], a recent longitudinal study failed to confirm that the exacerbation rate during longitudinal follow-up was associated with circulating or sputum eosinophil counts [33]. However, as expected, prior exacerbations increased the probability of subsequent exacerbations, and the observed exacerbation rates were also associated with older age, female sex, higher body mass index, worse asthma symptoms, lower spirometric function, higher doses of inhaled steroids, more frequent gastroesophageal reflux, nasal polyposis, diabetes, hypertension, depression, and oral-steroid treatment. Interestingly, neutrophils, and IL-6 concentrations, indicative of non-Type2 asthma, were highest in the exacerbation-prone patients [33].

Alternatively, a composite sputum 6-gene biomarker signature (6GS) provided by sputum transcriptomic analysis, which included Charcot-Leyden crystal galectin *[CLC]*, carboxypeptidase 3 *[CPA3]*, deoxyribonuclease 1-like 3 *[DNASE1L3]*, alkaline phosphatise, liver/bone/kidney *[ALP1]*, *CXCR2* and *IL1*B, was a significantly better predictor of future frequent severe exacerbations in patients with moderate-to-severe asthma than blood eosinophils. The 6GS signature also predicted airway inflammatory phenotype, defined as;

eosinophilic asthma (sputum eosinophil count ≥ 3%), neutrophilic asthma (sputum neutrophil count ≥ 61%), mixed granulocytic asthma (sputum neutrophil count ≥ 61% and eosinophil count ≥ 3%), and paucigranulocytic asthma (sputum neutrophil count < 61% and eosinophil count < 3%), and response to corticosteroid therapy [34, and references therein].

Confounding factors in the determination of peripheral eosinophil counts may be the high variation in eosinophil counts with a diurnal rhythm and also on a week-to-week basis [35], which are significantly reduced with ICS and systemic corticosteroid treatment.

A recent systematic review of the literature confirmed that oral corticosteroid therapy in stable asthma improves lung function and patient reported symptoms, while reducing markers of Type2 inflammation including blood and sputum eosinophil counts and FeNO. Additionally, higher levels of these Type2 biomarkers at baseline were predictive of a greater response to OCS [23]. Further, the greater reduction in blood (76% (95% CI: 63, 88)) and sputum (89% (95% CI: 79, 98)) eosinophils than FeNO (35% (95% CI: 28,41)) confirmed that OCS is a more potent inhibitor of the IL-5 pathway than the IL-4/IL-13 pathway [23] (see the section on Type2 cytokines below).

It was suggested that alignment of OCS therapy with an easily measured biomarker could lead to more targeted treatment and better patient outcomes [23]. Such a biomarker-driven approach potentially reduces the risk of adverse effects associated with over-prescribing OCS [36]. The results of a recent randomised control trial confirmed that a composite Type2 biomarker (FeNO, blood eosinophil count, serum periostin)-driven treatment strategy to reduce steroid dose was more effective than a symptom-based approach in the 40% of patients who followed the treatment advice [37]. However, the study revealed that many patients in both groups were, unexpectedly, reluctant to reduce ICS treatment.

Patients with severe asthma who do not respond to standard care or have significant residual impairment in lung function following OCS treatment [23] have been the focus of newly developed and emerging biologic, monoclonal antibody-based, add-on therapies [38, 39, 40]. The steroid-sparing effects of these highly-targeted therapies also reduce the risk of adverse effects associated with OCS [36]. Interleukin (IL)-5 is a Th2 cytokine that plays a central role in sustained eosinophilic inflammation in asthma (see section on cytokines, below) and anti-IL5 therapies are now approved by the FDA and EMA [40]. Successful outcomes for these new therapies depend on identifying sub-groups of patients likely to respond.

Thus, for example, sputum eosinophilia (>3%) was used to select patient groups in clinical trials of anti-IL-5/anti-IL-5R biologic therapies that showed reduction in eosinophil counts and asthma exacerbations [reviewed in 25], results that are now translating into real world data [reviewed in 38].

Ideal patients for these therapies have been proposed to have blood eosinophil counts ≥300/µl for anti-IL-5 (mepolizumab, reslizumab) and ≥400/µl for anti-IL-5 receptor α (benralizumab) therapies [40]. Recent ERS/ATS guidelines now suggest using anti-IL-5 therapies for severe uncontrolled adult eosinophilic asthma phenotypes, using a blood eosinophil cut-point ≥150/µL to guide anti-IL-5 initiation in adult patients with severe asthma [41]. However, in the real-world setting, although anti-IL-5 therapy (mepolizumab) was effective in patients with severe asthma, and reduced exacerbations and OCS use and improved FEV1 above the minimum clinically important difference (MCID) [41], the peripheral blood eosinophil count at baseline was not related to the magnitude of the clinical/functional response and a predictive value of baseline blood eosinophil count could not be detected in this real-life setting [42].

Serum IgE

IgE is central to the pathophysiology of allergic asthma. However, there is considerable overlap in total IgE levels between atopic and non-atopic individuals, making it difficult to identify atopy [43]. The evaluation of serum total IgE or allergen-specific IgE has not provided evidence that either of these measures are sensitive or effective biomarkers to predict response to anti-IgE therapy [43]. Not all patients with moderate-to-severe uncontrolled allergic asthma respond to Omalizumab, but high levels of FeNO, peripheral blood eosinophil counts and serum periostin may be useful biomarkers to predict patients who would benefit from Omalizumab therapy [44].

Recent ERS/ATS guidelines suggest considering specific eosinophil (≥260 /µL) and exhaled nitric oxide fraction (≥19.5 ppb) cut-offs to identify adolescents or adults with the greatest likelihood of response to Omalizumab anti-IgE therapy [41].

Cytokines in sputum and blood

A clinically useful non-invasive asthma biomarker ideally should accurately reflect airway inflammation, and if not the therapeutic target *per se*, it should be mechanistically linked to the therapeutic target, and be easily measured using validated standardised assays [25].

Detection of the therapeutic target would be the most direct approach to identify patients most likely to benefit from treatment. In this context, IL-5 and IL-4/IL-13 are key effector cytokines in Type 2 airway inflammation and play significant roles in the pathophysiology of asthma [reviewed in 11, 12, 45], including eosinophil recruitment and activation, and are targets for biologic therapies (Figure 2).

Interleukin-5

IL-5 plays a central role in eosinophilic inflammation associated with allergic and non-allergic Type2 asthma [11, 12]. IL-5 promotes the development and maturation of eosinophils in the bone marrow, and following migration into tissue sites of inflammation, IL-5 prolongs survival and enhances effector functions of eosinophils. Although IL-5 at physiological concentrations is not a potent chemoattractant for human eosinophils, it primes the chemotactic response of these cells.

IL-5 is generated by pathogenic effector Th2 cells (peTh2), an IL-5+ subset of conventional Th2 T-cells that arise following chronic antigen exposure [46], group 2 innate lymphoid cells (ILC2s), mast cells and natural killer T cells [reviewed in 47]. Thus peTh2 cells are proposed to drive eosinophilia in allergic diseases [46], while ILC2s, which secrete high levels of IL-5 and IL-13 as compared with other cell types, may play a vital role in eosinophilic non-allergic asthma. ILC2s expressing IL-5 mRNA are increased in the sputum of severe asthmatics despite the use of high-dose inhaled corticosteroids (ICS) and thus could be important cellular sources of IL-5 in the airways of these steroid refractory patients [47] .

At sites of inflammation, the epithelial-derived cytokines IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) activate peTh2 cells and ILC2s and there is evidence for increased expression of these important regulatory cytokines in asthma [47], for which novel biologics are in development [38, 39].

Interleukin-4 and Interleukin-13

IL-4 and IL-13 induce expression of the endothelial adhesion molecule vascular cell adhesion molecule-1 (VCAM-1), an adhesive ligand for peripheral blood eosinophils, but not neutrophils, at sites of inflammation. IL-4 and IL-13 also stimulate expression of the eosinophil chemoattractants RANTES and eotaxin from epithelial cells, airway smooth muscle cells and airway fibroblasts, that with increased VCAM-1 expression, synergistically and effectively induce eosinophil migration into tissue [47].

IL-13 is a glycoprotein whose primary source is activated CD4+ TH2 and to a lesser extent TH1 cells, CD8+ T cells, mast cells, basophils, eosinophils, and natural killer Tcells [45]. Type 2 innate lymphoid cells (ILC2) have more recently been identified as a source of IL-13 responsible for bronchial epithelial tight junction leakiness and reduced barrier function [11, 48]. Cytokine production by ILC2 cells is resistant to steroid treatment and it is suggested that ILC2 cells play a significant role in more complex, steroid refractory, asthma [11]. This steroid refractory endotype is characterised by increased gene expression for IL-4, IL-13 and IL-5 by cells in induced sputum in patients with severe asthma despite ICS therapy [10, 11].

IL-13 has a broad spectrum of activities in asthma, including the switch from IgM to IgE synthesis by plasma cells, promotion of eosinophil recruitment to the lung through increased synthesis of eotaxin and other CC chemokines and upregulated expression of endothelial VCAM-1, a selectively adhesive ligand for eosinophils [47], reduced expression of epithelial tight junction proteins and breakdown of the epithelial barrier function, reduced secretion of the structural focal adhesion protein paxillin leading to sloughing of airway epithelial cells, goblet cell hyperplasia and increased mucus production, increased expression of inducible nitric oxide synthase (iNOS) by airway epithelial cells and therefore increased fraction of exhaled nitric oxide (FeNO) in asthma, transformation of airway fibroblasts to myofibroblasts leading to collagen deposition, proliferation of airway smooth muscle, and stimulation of airways hyperresponsiveness [11, 45, 48].

In addition, periostin, an extracellular matrix protein expressed by airway epithelial cells in response to IL-13 and highly expressed in the airways of asthmatics, further upregulates the effector functions of eosinophils [47]. Conversely, IL-13 inhibits expression of the cytoskeletal protein ezrin in airway epithelial cells contributing to epithelial cell damage [49]. Down-regulation of ezrin potentially interferes not only with epithelial barrier function, but membrane protein localisation, including the cystic fibrosis transmembrane conductance regulator (CFTR) protein, cell polarisation and differentiation of cilia [50]. It is of interest, therefore, that CFTR dysfunction has been implicated in airway hyperreactivity in asthma [51]. Ezrin was detected in bronchial epithelial exosomes [49]. The production of exosomes is enhanced by IL-13 [52] and these membrane-bound vesicles are found in exhaled breath condensate [53]. Serum ezrin levels were negatively correlated with serum IL-13 and periostin, and the lower levels of ezrin detected in exhaled breath condensate and serum in asthma was highly significantly correlated with decline in lung function and might be a

potential biomarker of loss of asthma control [49] (and see the section below on biomarkers of asthma in exhaled breath).

Serum IL-5 and IL-13 were shown to be equivalent as best predictors of blood eosinophilia and cytokine levels remain stable over a 6-week period [29], unlike peripheral eosinophil counts which, in healthy adults, vary with a diurnal rhythm and on a week-to-week basis [35] and decrease with inhaled and oral corticosteroid therapy. IL-5 and IL-13 selected an eosinophilic subendotype that was less severe and was associated with AHR. Increased asthma severity was associated with increased serum levels MCP-1 and IL-8 (the mixed endotype). It was suggested that selecting patients based on those biomarkers alone, or combined with FeNO or blood eosinophils, might prove useful to select a blood eosinophilia sub-endotype responding to asthma treatment [29].

However, serum IL-5 [54] and IL-13 [55] are reported to be poor indicators of disease activity in acute asthma, and not different to healthy control levels.

In the case of IL-13, this cytokine is produced in the inflamed airway where bronchial epithelial cells and fibroblasts express high levels of IL-13 receptors. The IL-13Rα2 has been considered a decoy receptor that binds free IL-13 strongly and internalises IL-13 from extracellular spaces [56]. The resulting levels of systemic free IL-13 are correspondingly low and not significantly different between patients with symptomatic and asymptomatic asthma and healthy controls [55]. Thus IL-13 is not a biomarker of asthma, but is mechanistically linked to the stimulation of iNOS expression and periostin synthesis by bronchial epithelial cells, and was considered a potential therapeutic target [11, 12, 45]. Thus FeNO, an indicator of iNOS expression and activity, reflects IL-13 activity and a decrease in FeNO is reported amongst the responses to anti-IL-13 therapy in asthma [57, 58, 59]. However, despite evidence for effects on the target, especially in patients with high serum periostin or FeNO levels at baseline which predict response to therapy, an inconsistent effect on the rate of exacerbations in patients with severe asthma [58, 60] has led to the discontinuation of anti-IL-13 therapies.

To varying degrees, IL-4 has similar activities to IL-13 through binding to the same IL-4Rα receptor protein, and signalling through STAT6 [11, 12]. In addition, IL-4 has unique properties related to differentiation of naïve CD4+ T helper cells to a Th2 phenotype. The combined anti-IL-4/IL-13 therapeutic approach, for example with the monoclonal antibody

Dupilumab which binds the IL-4Rα and inhibits both IL-4 and IL-13 signalling pathways, is more effective than targeting IL-13 alone. Studies showed that over 52 weeks in patients with uncontrolled moderate-to-severe asthma Dupilumab significantly improved lung function, reduced Th2 inflammation, oral corticosteroid use and exacerbations, and the effects were greater in patients with higher FeNO (>25 ppb) and blood eosinophils (≥300 cells/µl), or FeNO >25ppb and blood eosinophils ≥ 150 cells/µl at baseline, biomarkers that predict response to therapy [61, 62]. Dupilumab is now approved by the EMA and FDA as an add-on therapy for the treatment of patients with uncontrolled severe asthma and Type2 inflammation characterised by raised blood eosinophils and/or FeNO who remain uncontrolled with high dose ICS plus another maintenance medication [63].

However, recent ERS/ATS guidelines for the management of severe asthma suggest using Dupilumab as an add-on therapy for adult patients with severe eosinophilic asthma and for those with severe corticosteroid-dependent asthma *regardless* of blood eosinophil counts [41].

Biomarkers of asthma in exhaled breath.

Exosomes

Exosomes, membrane-bound microvesicles, are found in exhaled breath condensate and, as described above, have been reported to enclose the epithelial cytoskeletal protein ezrin, which is reduced in asthma and represents a potential biomarker of loss of asthma control [49].

It has also been suggested that exosome-enclosed microRNAs (miRNAs) in exhaled breath condensate (EBC) hold potential for biomarker discovery in patients with pulmonary diseases [53]. MicroRNAs (miRNAs) are small non-coding RNAs comprising 22–25 nucleotides that are widely expressed in a variety of mammalian cells. The nucleotide sequence of miRNA is complementary to that of the target mRNA (typically 3′ UTR) and, via base-pairing, miRNAs initiate the process of gene silencing either by destabilizing the mRNA or by degradation of mRNA targets. Changes in the expression of miRNAs and post-transcriptional gene regulation following cell stimulation can lead to significant modulation of target protein expression and function potentially contributing to disease pathogenesis in asthma [reviewed in 64]. Micro-RNA expression has been linked to multiple cellular and molecular mechanisms in asthma, including regulation of ILC2 biology, steroid refractory asthma and

impaired anti-viral immune defences [reviewed in 65]. These miRNAs are proposed to be biomarkers of gene dysregulation in asthma and modifiable therapeutic targets, and have been described in epithelial cells, eosinophils, airway smooth muscle cells, blood and sputum [64, 65].

Comparing the miRNA profile in EBC from patients with asthma and healthy controls identified 11 differentially expressed miRNAs, of which the lower expression of hsa-miR-556-5p in asthma was most significant [53]. Hypothetically, lower expression of hsa-miR-556-5p in the airways in asthma may relate to increased expression of the transcriptional co-activator Yes-Associated Protein (YAP) [66]. The aberrant activation of YAP is implicated in various lung diseases, including asthma [67]. YAP is increased in bronchial smooth muscle and stimulates smooth muscle proliferation in mouse models of asthma [68] and the migration and proliferation of foetal airway smooth muscle cells [69]. In support of this hypothesis is the observation of significantly higher levels of YAP mRNA in the induced sputum of children with newly diagnosed, untreated, mild asthma versus healthy control children, and further significantly higher levels in moderate disease [70].

As a non-invasive source for biomarker discovery, the majority of miRNAs in EBC are found in exosomes in a stable form suitable for further potential biomarker discovery [53].

Exhaled hydrogen peroxide.

Oxidative stress reflects the imbalance between endogenous anti-oxidants and the production of reactive oxygen and reactive nitrogen species in cells and tissues during inflammatory processes, and is a feature of asthma associated with airflow obstruction, airway hyperreactivity and remodeling [71]. Activated inflammatory cells, particularly neutrophils and eosinophils, respond with a respiratory burst, leading to increased production of reactive oxygen species (ROS), with enzyme-catalysed dismutation of superoxide anions to hydrogen peroxide. As H_2O_2 is released by the activation of inflammatory cells, including neutrophils and eosinophils, EBC levels of H_2O_2 might reflect the influx of inflammatory cells into the lungs as well as disease severity, as indicated by a significant decrease with ICS therapy [72].

Systematic reviews have indicated that concentrations of exhaled hydrogen peroxide, a marker of oxidative stress, in exhaled breath condensate (EBC) are elevated and related to lower lung function tests in adults with asthma compared to healthy subjects [72] and elevated in children with asthma not receiving anti-inflammatory treatment [73].

Although the concentration of H_2O_2 in exhaled air has been reported to be elevated in asthma, results are inconsistent and difficult to reproduce. This might reflect the fact that H_2O_2 concentration in a concentrate of ambient air is significantly higher ($p<0.001$) than in EBC, and may contribute to the heterogeneity and limited reproducibility of study results [74]. It was concluded that a valid determination of endogenous H_2O_2 production requires inhalation filters.

Analysis of reactive oxygen metabolite levels, a measure of hydroperoxides, in serum was shown to be predictive of the risk of severe exacerbations of asthma [75]. However, assessment of EBC markers is a non-invasive approach to evaluate airway inflammation, exacerbations, and disease severity of asthma, and to monitor the effectiveness of anti-inflammatory treatment regimens.

In this respect, devices, such as the battery operated, handheld, Inflammachek device have been developed for point of care measurement of H_2O_2 level in exhaled breath, but remain to be validated [76].

Fractional exhaled nitric oxide (FeNO)

Non-invasive means of assessing airway inflammation presently include the measurement of fractional exhaled nitric oxide (FeNO), a volatile biomarker in expired air. Nitric oxide (NO) in orally exhaled air mainly originates from the respiratory epithelium, but is also produced by activated macrophages and eosinophils, and is increased in steroid-naive asthma patients and during an exacerbation [reviewed in 77]. NO is produced by inducible NO synthase (iNOS) and has multiple effects in the inflamed airway, including vasodilation in the pulmonary vasculature, promoting inflammation and immune defence. Expression of iNOS, but not other forms of NOS, and synthesis of NO is rapidly inhibited by inhaled and oral corticosteroids [77]. Measurement of exhaled NO was therefore proposed to be a simple non-invasive method of measuring inflammation in asthma, as well as monitoring response to corticosteroid therapy.

In patients with asthma, iNOS expression is upregulated by IL-4 and IL-13 via the activation of STAT-6 in the bronchial epithelium. Thus, exhaled NO primarily signals Type2-driven inflammation in the bronchial mucosa [78]. Therefore, as described above, in patients treated with Dupilumab, which targets the IL-4/IL-13 pathway, FeNO is able to predict treatment

response and patients with a higher FeNO have the greatest reduction in exacerbations [61, 62].

FeNO correlates with sputum eosinophilia [79] and is a predictor for eosinophilic airway inflammation [26]. Wagener et al. (2015) showed that FeNO levels ≥42 parts per billion had a positive predictive value of 74% for sputum eosinophilia of >3%, an accepted cut-off for airway eosinophilic inflammation by sputum cell count [26]. Additionally, FeNO levels predict steroid responsiveness [80]. Subjects with baseline FeNO levels > 47 ppb had significantly greater response to 4-weeks treatment with inhaled fluticasone, measured as increase in FEV_1, increase in mean morning peak flows, improved respiratory symptoms, and reduction in airway hyperresponsiveness to adenosine monophosphate (AMP) [80].

FeNO does not measure neutrophilic airway inflammation, which is a recognized component of steroid insensitive, severe persistent asthma [81]. As a consequence, FeNO cannot monitor management in the 20% of asthmatics with airway neutrophilic inflammation (>61% neutrophils in sputum) [82]. More recently, increased neutrophil counts were detected in bronchial tissue in more than 50% of patients with mild to severe asthma, and was associated with greater disease severity, functional residual capacity, inhaled corticosteroid (ICS) dose and exacerbations 148]. However, recent reports indicate that the prevalence of T2-low asthma may be as low as 5% [37].

Conventional tests such as FEV1 reversibility or provocation tests are only indirectly associated with airway inflammation in asthma. Dweik et al. (2011) concluded that the FeNO measurement offers added advantages for patient care including, but not limited to (1) detecting of eosinophilic airway inflammation, (2) determining the likelihood of corticosteroid responsiveness, (3) monitoring of airway inflammation to determine the potential need for corticosteroid, and (4) unmasking of otherwise unsuspected non-adherence to corticosteroid therapy in patients with mild-to-moderate asthma [83].

Recent systematic reviews found that FeNO guided asthma management showed a statistically significant reduction in exacerbations of any severity, but there was no statistically significant benefit in terms of severe exacerbations or inhaled corticosteroid use [84, 85]. Essat et al. (2016) concluded that although FeNO testing for adult asthma management may confer clinical benefit, further research is needed to establish its role [84]. Specifically, it was suggested that further research is needed to clearly define which management protocols (including FeNO cut-off points) offer best results and which patient

groups would benefit the most [84]. Additionally, in view of the strong circadian rhythm of FeNO in asthma, with lower levels detected during the night than during the day, the importance of time of day in diagnostic sampling and effects on diagnostic algorithms is highlighted [86]. Other confounding factors in the measurement of FeNO, include age, height, weight, atopy, race, smoking and gender [87]. Petsky et al. (2018) concluded there is insufficient evidence to advocate the use of FeNO analysis in routine clinical practice [85].

Scottish Intercollegiate Guidelines Network/British Thoracic Society (SIGN/BTS) guidelines consider that a positive FeNO test (>40 ppb in adults, >35 ppb in children) is indicative of eosinophilic inflammation and provides supportive, but not conclusive, evidence for an asthma diagnosis, while a negative test does not exclude asthma [88]. However, in view of the findings of systematic reviews [84, 85], the SIGN/BTS guidelines consider there is insufficient evidence to recommend the routine use of FeNO testing for the monitoring of asthma in adults or children.

FeNO has now been integrated into National Institute for Health and Care Excellence (NICE) national asthma guidelines for both diagnosis and management. NICE guidelines regard a FeNO level of 40 parts per billion (ppb) or more as a positive objective diagnostic test in adults, or 35 ppb in children, in addition to detailed history and spirometry [89]. It is not recommended to routinely use FeNO to monitor asthma control, but FeNO measurement may be an option to support asthma management in people who are symptomatic despite using inhaled corticosteroids [89].

ATS clinical practise guidelines recommended that FeNO greater than 50 ppb (> 35 ppb in children) be used to indicate that eosinophilic inflammation and, in symptomatic patients, responsiveness to corticosteroids are likely, and FeNO may be used to support the diagnosis of asthma in situations in which objective evidence is needed [83]. However, ATS/ERS guidelines do not recommend that clinicians use FeNO to guide therapy in adults or children with severe asthma, based on the uncertain benefit from monitoring FeNO [90].

Volatile organic compounds (VOCs)

Breathomics is the application of metabolomics to exhaled breath samples, which contain low molecular weight volatile organic compounds (VOCs) derived from cellular processes occurring either locally in the airway or systemically. The profile and concentration of

exhaled metabolites alters with age and with the circadian rhythm, and with disease compared to health [86]. Normal exhaled breath is estimated to contain more than 3,400 different VOCs [91]. Although each breath may contain less than 250 VOCs, the VOC composition is highly variable between individuals, depending on the exposome of the individual containing VOCs of environmental origin, and physiological factors [92, 93]. Nevertheless, the breath profile is relatively stable within individuals.

There is evidence to propose a number of exhaled VOCs as biomarkers of asthma, inflammatory phenotype, disease activity and adherence to therapy [94, 95], although there is little evidence of their use to monitor response to therapy [94, 95]. Low molecular weight VOCs are carbon-containing metabolites that may be identified by advanced gas chromatography/mass spectrometry (GC-MS) techniques, including two-dimensional GC-MS [92]. Using these techniques, Schleich et al [96] identified surrogate biomarkers for neutrophilic and eosinophilic asthma. The combination of nonanal, 1-propanol, and hexane identified neutrophilic inflammation (≥ 76% neutrophils in induced sputum), identifying a novel biomarker of neutrophilic asthma. The levels of 3,7-dimethylnonane, 1-propanol, and nonanal were on average 4.6 times, 3.4 times, and 1.5 times, respectively, higher in the neutrophilic than in the eosinophilic inflammatory subtype. Lower concentrations of hexane and 2-hexanone identified eosinophilic asthma (≥ 3% eosinophils in induced sputum). The prediction accuracy of sputum eosinophilia using VOCs measurement was comparable with the accuracy of blood eosinophil counts and FeNO. Further, the combination of FeNO, blood eosinophil count, and VOCs was highly predictive of increased sputum eosinophil counts with an AUROC of 0.87.

Brinkman et al [95] investigated the association of exhaled VOCs analysed using gas chromatography coupled with time-of-flight mass spectrometry in severe asthma patients with urinary levels of salbutamol and OCSs analysed by liquid chromatography coupled with high-resolution mass spectrometry. They linked exhaled VOCs to urinary detection of salbutamol and OCSs, indicating an analytical approach for monitoring adherence to therapy through the non-invasive analysis of breath biomarkers.

Alternatively a VOC profile, without identification of component VOCs, may be used to recognise disease, for example using the electronic nose (eNose) technology, more suited to clinical point-of-care testing [92]. In their proof-of-concept study, Plaza et al [97] showed that different inflammatory asthma phenotypes based on induced sputum analysis can be

identified by their breath-profiles using eNose technology. In patients with persistent asthma, the eNose distinguished breath-profiles from asthma phenotypes classified as neutrophilic (>61% neutrophils), eosinophilic (>3% eosinophils), or paucigranulocytic (<61% neutrophils and <3% eosinophils) according to the inflammatory cell profile in induced sputum. It was therefore proposed that eNose may be a simple alternative to identify airway inflammatory phenotypes in clinical practice.

A recent meta-analysis confirmed there is consistency across the literature that exhaled VOCs are sensitive to underlying inflammation in adult asthma [98]. Further, the recently established human breathomics database [99] is likely to aid in the identification and further investigation of potential biomarkers in the breath of asthma patients.

Urinary metabolites.

The advantages of measuring potential biomarkers in urine are that sampling is non-invasive, requires minimal patient effort, and samples can be collected across all ages. The disadvantages, however, are that samples are dilute and biomarker concentrations might be difficult to measure, biomarker normalization standards are lacking and biomarker results might not be repeatable or might not reflect changes in the airway [reviewed in 100]

Eicosanoids.

Kolmert et al (2021) recently reported the results of the analysis of urinary metabolites of prostaglandins (PGs), cysteinyl leukotrienes (cysLTs) and isoprostanes in the U-BIOPRED (Unbiased Biomarkers for the Prediction of Respiratory Diseases Outcomes) study including 86 adults with mild-to-moderate asthma, 411 with severe asthma, and 100 healthy control participants [101]. Samples were analysed using electrospray ionization mass spectrometry. Higher concentrations of urinary LTE4, the stable end-product of cysLT metabolism, were measured in mild-to-moderate asthma than in healthy controls, and these were further significantly elevated in severe asthma. Urinary PGD2 metabolites were higher in severe asthma than in mild-to-moderate asthma, and higher than levels in samples from healthy control samples [101].

High concentrations of urinary LTE4 and PGD2 metabolites were associated with lower lung function and increased amounts of exhaled nitric oxide and eosinophil markers of Type2 inflammation in blood, (eosinophils, serum periostin and IL-13) and sputum (eosinophils) in U-BIOPRED participants and in a group of adolescents with severe asthma or controlled

persistent asthma. The eicosanoid signature and its relationship to Type2 inflammation was stable over 12-18 months and, rather unexpectedly, the use of oral corticosteroids did not influence urinary eicosanoid metabolite concentrations.

In contrast to the associations for PGD2 and LTE4 with Type2 asthma, high urinary isoprostane concentrations were associated with the characteristics of a different phenotype. This group was enriched in women with high BMI, lower FENO, poor quality of life (AQLQ), more frequent exacerbations, elevated hsCRP, and less asthma control. This observation suggests a role for urinary isoprostanes as markers of a non-Type2 phenotype, presumably the phenotype of asthma dominated by women with a high BMI [101].

Leukotrienes and prostaglandins are lipids produced via the action of 5-lipoxygenase and cyclooxygenase enzymes, respectively, on arachidonic acid metabolism. CysLTs play a central role in asthma pathophysiology. They induce bronchoconstriction, mucin production, plasma exudation and inflammatory cell recruitment [102] and are produced through IgE-dependent and IgE-independent mechanisms by activated mast cells [103], basophils [104] and eosinophils [105] at sites of inflammation in asthma. Urinary LTE4 levels were significantly associated with FEV1 responses to montelukast, leukotriene receptor antagonist (LTRA), step-up therapy [106], and high urinary levels of LTE4 may be a biomarker to select patients who respond to LTRA therapy [106, 107].

PGD2, a key player in airway inflammation, is synthesised by mast cells, dendritic cells, and Th2 cells [108], notably the IL-5+ve pathogenic effector Th2 cells [46]. Activation of the PGD2 receptor, DP2 (also known as the chemoattractant receptor homologous molecule expressed on Th2 cells (CRTH2)), which is expressed on the membrane surface of T_H2 cells, ILC2 cells, mast cells and eosinophils, induces significant cytokine synthesis by Th2 and ILC2 cells, in addition to eosinophil chemotaxis, activation and degranulation, contributing to airway inflammation in asthma [108]. Importantly, since LTE4 enhanced the activation of ILC2 and Type2 cytokine production by PGD_2 [109], there may also be synergistic effects of PGD_2 and cysLTs in promoting inflammatory responses.

It was suggested that many older patients with severe asthma and persistent steroid refractory airway Type2 inflammation would benefit from alternative anti-inflammatory therapy, such as inhibitors of the prostaglandin D_2 receptor 2 [10]. However, although fevipiprant, an oral antagonist of the prostaglandin D_2 receptor 2, reduced sputum eosinophils and improved lung

function in phase 2 trials of patients with asthma, Phase III trials showed no significant reduction in asthma exacerbations in patients with severe asthma [110].

Thus monitoring of urinary eicosanoids can identify Type2 and non-Type2 phenotypes in asthma and this approach provides a new non-invasive approach for molecular phenotyping of adult and adolescent asthma. Validated techniques for the extraction and quantitative analysis of thirty two urinary eicosanoids by LC-MS/MS are available, and suitable for focused mechanistic studies as well as large-scale clinical and epidemiological studies [111]. Future clinical trials will be important in determining how urinary eicosanoid profiles can be used to direct precision treatments.

Urinary bromotyrosine

Bromotyrosine is formed from post-translational modification of protein tyrosine residues by the brominating oxidant hypobromous acid, which is generated uniquely via the activity of eosinophil peroxidase in activated eosinophils during the respiratory burst. Bromotyrosine is a stable end-product of modified protein degradation resulting in airway and urinary concentrations that are higher in patients with allergic asthma and increase during an asthma exacerbation. Urinary bromotyrosine levels are associated with airflow limitation, asthma control and risk of future exacerbations [reviewed in 71, 112]. Studies have shown that urinary bromotyrosine concentrations are decreased with inhaled corticosteroid treatment and high baseline values predict greater corticosteroid responsiveness. However, concordance among sputum eosinophil counts, FeNO concentration and urinary bromotyrosine concentration is not very high. Therefore the utility of bromotyrosine in the clinical setting might be best when assessed as a part of a larger panel of inflammatory biomarkers [71, 100, 112].

Urinary markers of coagulation

We recently reported increased levels of urinary fibrinopeptide-A (FP-A), a marker of the initiation of coagulation, 4-5 days prior to the on-set of asthma exacerbation [113]. We previously demonstrated that pulmonary coagulation is a feature of severe asthma [114] but it remains to be investigated whether measures of urinary FP-A provide a non-invasive biomarker of disease severity predictive of acute exacerbations.

Discussion

Biomarkers are critical for studies of disease pathogenesis, for the identification of different phenotypes and endotypes and monitoring the response to standard treatment or new targeted therapies for severe asthma, as evidenced by the development of novel biologic therapies targeting Type2 inflammation. Biomarkers complement symptom scoring and lung function measurements in asthma management. The challenge is to better understand non-Type2 mechanisms and develop new treatments and management strategies in non-Type2 severe asthma. Identification of biomarkers of this endotype, as indicators of pathogenic processes or response to an intervention, will play a role in these investigations.

The development of multiple 'omics platforms, notably transcriptomics [7], but also breathomics and urinary metabolomics, as described above, have led to an increasing appreciation of the biological complexity of asthma pathogenesis. Multiplication in the number of different endotypes being recognised, emphasises the need not only for detailed composite molecular fingerprints to identify these endotypes and direct novel therapeutic approaches with precision, but for improved methodologies that deliver such refinement in the detail of composite molecular fingerprints (biomarkers) through non-invasive methodology, of which analysis of breath VOCs and urinary eicosanoid profiles stand out as potential candidates. Because of their non-invasive nature, the measurement of breath VOCs and urinary eicosanoids would be particularly useful at the point of primary care where most asthma patients are managed.

Advancements in MS instrument design in terms of both simplification and size without compromising sensitivity and specificity, indicate that such miniaturized MS instruments could be used in diagnostic point-of-care tests. For example, with appropriate MS instrumentation and user-friendly interfaces for automated analysis, ambient ionization techniques [115] could provide quantitative point-of-care measurements of biomarkers in asthma.

Legends to Figures

Figure 1. Asthma phenotypes, endotypes and targeted treatment approaches in severe asthma. *Suggested biomarkers to evaluate treatment response of targeted therapy are complementary to the evaluation of the clinical response evaluation such as asthma exacerbation rate, asthma control, and/or asthma quality of life. **For evaluation of therapy-resistant airway

obstruction and/or severe airway hyperresponsiveness. Dashed arrow: based on proof-of-concept studies for which additional pragmatic or head-to-head clinical trials are required. CD sens; basophil activation threshold. Reproduced with permission [5].

Figure 2. Immune mechanisms and targeted treatment options for severe asthma according to Type2 and non-Type2 endotypes, with bronchial thermoplasty indicated for structural abnormalities. Reproduced with permission [5].

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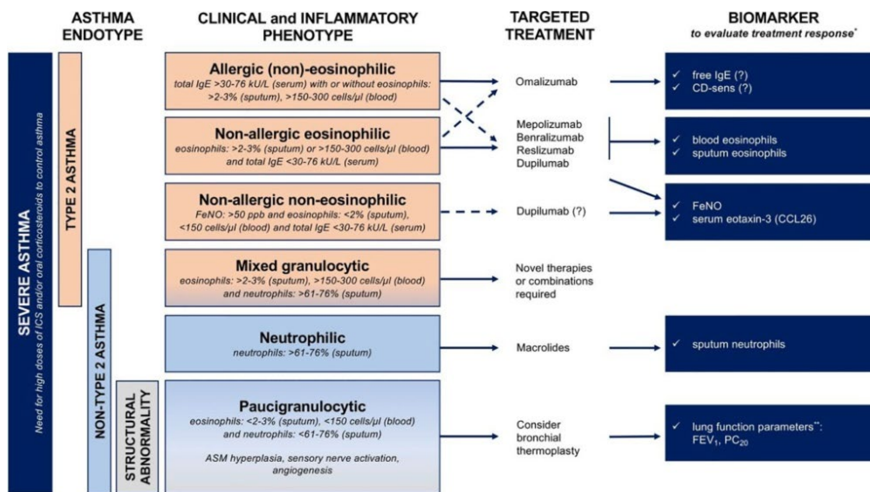

Figure 1. Asthma phenotypes, endotypes and targeted treatment approaches in severe asthma. *Suggested biomarkers to evaluate treatment response of targeted therapy are complementary to the evaluation of the clinical response evaluation such as asthma exacerbation rate, asthma control, and/or asthma quality of life. **For evaluation of therapy-resistant airway obstruction and/or severe airway hyperresponsiveness. Dashed arrow: based on proof-of-concept studies for which additional pragmatic or head-to-head clinical trials are required. CD sens; basophil activation threshold. Reproduced with permission [5].

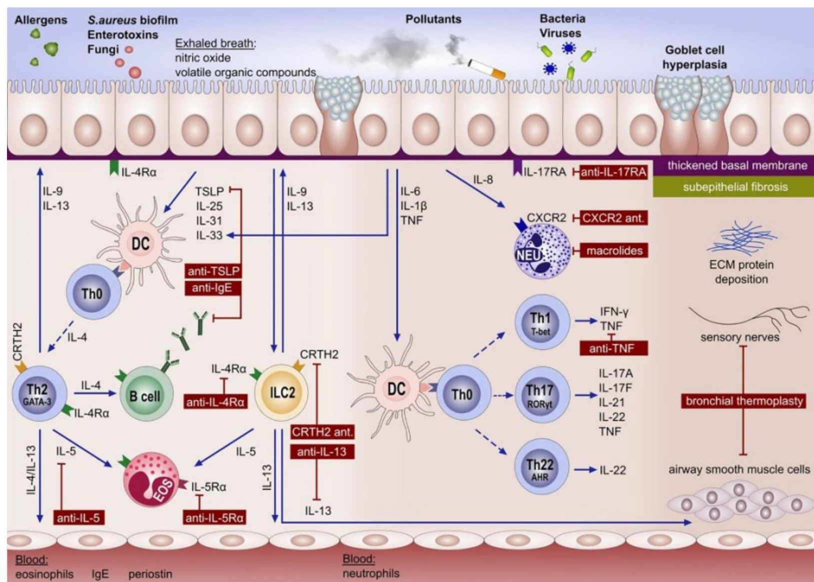

Figure 2. Immune mechanisms and targeted treatment options for severe asthma according to Type2 and non-Type2 endotypes, with bronchial thermoplasty indicated for structural abnormalities. Reproduced with permission [5].